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COMPLETE SPECIFICATION

Polymeric Colour Developers and their use in Detecting Coupling Compounds

We, MILES LABORATORIES, INC., a Corporation organised and existing under the laws of the State of Indiana, United States of America of 1127 Myrtle Street, Elkhart, Indiana, United States of America, do hereby declare the invention for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

This invention relates to the determination of coupling compounds. In one of its more particular aspects, this invention relates to the determination of aromatic hydroxyl and amino compounds and other compounds which undergo a visible change in coupling reactions with diazonium salts.

A number of methods for determining coupling compounds are available. These include both instrumental and chemical methods. In general, the instrumental methods are too complex to be used except by those who have had formal training in the use of them. Most chemical methods also require a relatively high degree of skill.

One of the most popular chemical methods for the determination of this class of compounds is that involving the coupling of an aromatic diazonium salt with reactive coupling compounds such as, for instance, phenols or aromatic amines or compounds containing active methylene or active methyl groups.

Use of this method enables the determination of phenols or aromatic amines by simple colorimetric techniques. However, the diazonium reaction is one which must be conducted under carefully regulated conditions of temperature and pH, and so involves a correspondingly high level of care in successfully carrying out the reaction.

It is accordingly an object of this invention to provide a method for determining reactive coupling compounds which involves none of the shortcomings of the prior art.

[*Price 4s. 6d.*]

It is another object of this invention to provide such a method which is convenient to use and which does not require a high level of training and skill in the operation of analytical instruments or the carrying out of complex chemical reactions.

Another object of this invention is to carry out the above mentioned determination in a very short time and without special equipment.

Another object of this invention is to provide a method for determining reactive coupling compounds when they are present in very low concentrations.

Other objects and advantages of this invention will become apparent in the course of the following detailed disclosure and description.

It has now been found that a convenient test for reactive coupling compounds can be provided by using an insoluble colour developer. Such developer can be prepared by diazotizing a polymeric material containing free aromatic amino groups.

The most obvious method for providing free aromatic amino groups attached to a polymer backbone is by the nitration of benzenoid units attached to a polymer such as a polystyrene, followed by reduction of the resulting nitro groups to amino groups. The aromatic amino groups resulting can then be readily diazotized by treatment with nitrous acid or other diazotizing agent usually by reaction with a dilute solution of sodium nitrite and an acid such as hydrochloric acid. This method is disclosed in U.S. Patent No. 2,274,551 to William O. Kenyon, Louis M. Minsk and George P. Waugh. This latter method, however, is subject to the disadvantage that the nitration step, which involves the use of an oxidizing agent, causes a discoloration of the products which does not disappear completely upon reduction and subsequent diazotization. In contrast thereto the method of the present invention does not in-

volve the use of oxidizing agents and results in the provision of polymeric diazonium salts of the same colour as the amine from which they derive. These salts are capable of effective utilization as insoluble colour developers.

In order to provide such polymeric diazonium salts, one starts with a polymeric material which contains acid groups such as carboxylic, sulphonic or phosphoric acid groups. Such materials are commercially available in the form of a wide variety of cationic ion exchange resins. These resins can be reacted with a halogenating agent, for example, a thionyl halide such as $SOCl_2$, or a phosphorus halide such as PCl_3 or PCl_5. Alternatively, sodium or potassium salts of such resins can be reacted with $POCl_3$ or SO_2Cl_2. For example, in the case of a carboxylic acid cation exchange resin, reaction with thionyl chloride produces the acyl chloride of the resin as shown in the following equation:

$$\text{Resin-COOH} \xrightarrow{SOCl_2} \text{Resin-COCl}$$

The resin halide can then be reacted with an aromatic polyamine in water or in an anhydrous organic solvent in order to provide a resin amide in which at least one aromatic amino group remains free. For example, using such aromatic diamines as benzidine, o-tolidine, di-o-anisidine, the phenylenediamines (ortho, meta or para), the resin halide is converted to a resin amide which contains a free aromatic amino group in accordance with the following equation:

$$\text{Resin-COCl} + NH_2\text{—Ar—}NH_2 \longrightarrow \text{Resin-CO—NH—Ar—}NH_2 \cdot HCl$$

wherein Ar represents the aromatic nucleus to which the two amino groups shown are attached. Such aromatic nucleus may be monocyclic, that is benzenoid, or polycyclic as in the case of derivatives of naphthalene, biphenyl, anthracene or phenanthrene.

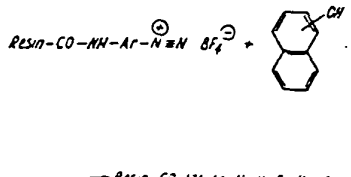
The resulting resin amide containing free aromatic amino groups can be diazotized to produced resin diazonium salts which can react with coupling compounds to give coloured azo products.

The diazotization can be performed, for example, by reacting the resin amide with sodium nitrite in the presence of an acid such as hydrochloric, sulphuric, phosphoric or fluoboric acid, resulting in the formulation of a polymeric diazonium salt. These diazenium salts are highly insoluble in aqueous media because of their polymeric nature and stable because of the presence in the resin of unreacted acid groups. This reaction can be illustrated as follows:

$$\text{Resin-CO—NH—Ar—}NH_2 \xrightarrow[HBF_4]{NaNO_2} \text{Resin-CO—NH—Ar—}\overset{\oplus}{N}\equiv N\ \overset{\ominus}{BF_4}$$

These polymeric diazonium salts can be stored for extended periods of time and used in aqueous solution to test for any compound which will couple with them. In particular these polymeric diazonium salts are useful as insoluble colour developers in the analysis of aromatic hydroxyl and amino compounds and other compounds which couple with diazonium salts to produce azo derivatives which are highly coloured.

This coupling reaction can be illustrated with a α-naphthol or β-naphthol by means of the following equation:



$$\longrightarrow \text{Resin-CO-NH-Ar-N=N-}C_{10}H_6\text{-CH}$$

The polymeric diazonium salts of this invention react with a wide number of coupling compounds such as aromatic amines, phenols and naphthols, naphthoic acid derivatives, nitro derivatives of amines and phenols, nitro-paraffins, substances with reactive methylene and methyl groups such as acetoacetic esters, β-oxo glutaric acid or 5-pyrazolone derivatives, sulphonic acid derivatives of phenol and naphthol and sulphonic acid derivatives of aromatic amines.

The coupling reaction to form the coloured azo compound is generally a quantitative one, that is, a certain amount of polymeric diazonium salt will react with a fixed amount of a coupling compound under suitable conditions. Owing to this the polymeric diazonium salts of this invention can be used as insoluble colour developers for quantitative or semi-quantitative determination of coupling compounds in several ways.

These insoluble colour developers are particularly useful when used in reagent strips. For instance, the insoluble colour developer can be coated upon sheets of a bibulous cellulosic material, such as filter paper to provide sheets containing zones of colour developer.

Rather than merely coating cellulosic sheets, an especially convenient and elegant method involves the formation of fibrous colour developer sheets from an aqueous homogenized mixture of insoluble colour developer and cellulosic fibrous material. These fibrous colour developer sheets can then be used to prepare strips or discs of colour developer.

The insoluble colour developers of this invention can also be used in the form of tablets which can be prepared by mixing the insoluble colour developer with suitable tabletting materials, for example, powdered cellulose.

Strips can be either of a continuous or discontinuous type, that is, the areas containing colour developer may be continuous or alternated with inert areas. For instance, continuous strips can be formed by a whole reactive zone fixed to a support such as paper, cardboard, wood, glass fiber or plastic. Graduated scales referring to quantities of the product to be determined can be imprinted upon such support. In the discontinuous type, the reactive zones are alternated with non-reactive zones.

For testing, when strips are used, an ascending or descending chromatographic technique is adopted. A pre-determined amount of solution to be tested for the coupling compound is absorbed into the strips and the strips are then washed with water. The coupling compound, in contact with the insoluble colour developer, generates a colour. Since the coupling reaction is a quantitative one, the colour intensity will be determined by the chemical composition of the reactive area, that is, by the amount of insoluble colour developer present in the strip, while the extension of the coloured area, measured by means of the previously mentioned imprinted graduations, will be proportional to the amount of the coupling compound coming into contact with the reactive zone.

Coupling product concentration can be determined in the one zone continuous strip by means of the previously mentioned imprinted graduations. In the discontinuous type multiple zone strip the number of the zones in which a colour is developed will indicated the concentration of the coupling compound being determined. For example, by using a series of three zones containing the colour developer fixed to a bibulous carrier, it is possible to determine the amount of coupling compounds present by the extent to which the advancing solvent front of the test medium carries the coupling compound along the strip. That is, a lesser concentration of coupling compound may react with the colour developer present in the first zone but not with that present in the second or third zone. A somewhat higher concentration may react with that present in the first two zones and a still higher concentration with that present in all three zones.

When discs are used, a pre-determined amount of the liquid under examination is absorbed into the disc. The reaction between the colour developer and any coupling compound present takes place in the disc and a colour, the intensity of which is proportional to the amount of coupling compound present, is developed. The colour obtained is compared with a suitable colour chart and the amount of coupling compound present in the solution under examination is determined. Tablets are used in a similar manner.

In addition to using the insoluble colour developer of this invention to detect reactive coupling compounds, it is also possible to detect systems which are capable of producing reactive coupling compounds. Since a wide variety of enzyme systems are capable of catalyzing the liberation of coupling compounds by their precursors, these systems can be readily detected by using the insoluble colour developer of this invention together with an appropriate coupling compound precursor as a substrate for the enzyme to be detected. Representative examples of these enzymatic reactions are the following:

a. lipase: the substrate used can be naphthyl laurate, myristate or caprylate. Lipase hydrolyses the substrate liberating the naphthol which is the specific compound to be determined.

b. acetyl esterase: the substrate used can be acetyl naphthol or acetyl naphthylamine. The enzymatic hydrolysis liberates naphthol or naphthylamine which are reactive coupling compounds.

c. N-acetylberaglucosaminidase: using as substrate naphthyl-N-acetylglucosamine the enzymatic reaction releases naphthol.

d. transaminase (GOT): in GOT transaminations L-glutamate + α-oxalacetate are formed from L-aspartate + α-oxo-glutarate. The α-oxalacetate formed in this reaction can be determined by means of the insoluble colour developers of this invention because its active methylene group reacts with diazonium salts.

e. leucine aminopeptidase: using L-leucyl-β-naphthylamide as a substrate, the enzymatic reaction liberates β-naphthylamino.

f. acid and basic phosphatases: the various phosphatases catalyze the hydrolysis of aromatic phosphates to the corresponding aromatic hydroxyl compounds which can then be readily detected by means of the colour developer of this invention.

Many convenient methods can be used for detecting these systems. For example, a suitable substrate for such enzyme system can be impregnated into one zone of a bibulous carrier to which is joined a zone of colour developer. When the substrate zone is moistened with a solution containing a phosphatase, the enzymatic reaction proceeds resulting in the release of an aromatic hydroxyl compound. Washing with water brings the latter into contact with the insoluble colour developer in the adjacent zone giving a colour.

Another convenient method of using the insoluble colour developer of this invention for the detection of an enzyme system provides the enzyme substrate in a tablet form which can be added to the solution which is being

tested for the presence of the enzyme. The colour developer in the form of a bibulous strip or tablet can then be moistened with the resulting solution to cause the desired colour reaction to occur if the enzyme is present. Such a test method is convenient, for example, for testing milk for the presence of phosphatases in order to check the pasteurization. It is well known that pasteurization temporarily destroys phosphatase in milk and that the presence of phosphatase in pasteurized milk soon after pasteurization is a sign of inadequate pasteurizing temperature or of the presence of raw milk.

Preparation of resins for diazotising in accordance with the invention are now illustrated in procedures 1 to 12; the invention is illustrated by Examples 13 to 25.

PROCEDURE 1

A polymer of methacrylic acid cation exchange resin (Amberlite IRA 64 type; Amberlite is a registered Trade Mark), in the acid form, was milled into a fine powder (particle size varying in the range of 20—50 microns) and dried. Fifty grams (50 g.) of the obtained powder were refluxed in 200 ml. of thionyl chloride under stirring for 6 hours. The reaction mixture was then filtered and the resulting chlorinated resin washed with anhydrous toluene and dried in high vacuum. The so obtained chlorinated resin contained 0.98 meq. of —COCl per gram.

PROCEDURE 2

The above mentioned fine form of resin, having a particle size of 20—50 microns, was chlorinated for 3 hours under otherwise the same conditions as used in Procedure 1. The resulting chlorinated resin contained 0.66 meq. of —COCl per gram.

PROCEDURE 3

The above mentioned fine form of resin, having a particle size of 20—50 microns, was chlorinated for 9 hours under otherwise the same conditions as used in Procedure 1 and the resin contained 1.4 meq. —COCl per gram.

PROCEDURE 4

A polymer of methacrylic acid cation exchange resin (Amberlite IRA 64 type), in the acid form, was reduced, by milling, to a fine powder (particle size 1—5 microns), then dried. Chlorination took place according to the procedure indicated in Procedure 1 for the duration of 3 hours. The resulting chlorinated resin contained 0.84 meq. of —COCl per gram.

PROCEDURE 5

A polymer of methacrylic acid cation exchange resin (Amberlite IRA 64 type) was changed into the sodium salt and reduced by milling to a fine powder (particle size varying from 50 to 70 microns) and then dried. A quantity of 50 g. of resin sodium salt was refluxed for 6 hours in 100 ml. of anhydrous carbon tetrachloride and 100 ml. of sulphuryl chloride. The resin was filtered and washed with carbon tetrachloride and then dried. The resulting chlorinated resin contained 0.56 meq. of —COCl per gram.

PROCEDURE 6

A quantity of 10 g. of chlorinated resin prepared according to the method of Procedure 1 was poured into a solution of di-o-anisidine (3.3 g.) in 75 ml. of dry toluene. The resulting suspension was heated at 85°C. for 5 hours with stirring. The mixture was filtered and the resin was washed in batch with ethanol (100 ml.) from which it was possible to recover the excess of unreacted amine. The resin was then put into a chromatographic column and washed with 2N HBF, (200 ml.) and then with water at the neutral point of effluent liquid. The resulting resin acylated amine, analyzed for diazotizable nitrogen, showed 47 mg. of mono-linked di-o-anisidine per gram.

PROCEDURE 7

A quantity of 10 g. of chlorinated resin prepared according to Procedure 1 was poured into a solution of di-o-anisidine (3.3 g.) and triethylamine (10 g.) in 75 ml. of dry toluene. The resulting suspension was heated at 85°C. for 6 hours with stirring. The resin was washed according to Procedure 6 and then dried. In the resulting resin acylated amine, analyzed for diazotizable nitrogen, 61 mg. of mono-linked di-o-anisidine per gram were present.

PROCEDURE 8

A solution of dioanisidine dihydrochloride (4.3 g.) in 200 ml. of water was refrigerated at a temperature of 0.5°C. To this solution a quantity of 10 g. of the chlorinated resin from Procedure 1 was added. Stirring and refrigeration were continued while 30 ml. of 20% NaOH was slowly added up to a pH value of 11—12. After 14 hours of stirring and refrigerating, the suspension was centrifuged and the precipitate washed with methanol. The washings were saved for the subsequent recovery of unreacted amine. The resin was then washed with HBF, and dried according to Procedure 6. The resulting resin acylated amine, analyzed for diazotizable nitrogen, showed a quantity of 7.20 mg. of mono-linked di-o-anisidine per gram.

PROCEDURE 9

A solution of m-phenylenediamine · 2HCl (1.8 g.) in 50 ml. of water was refrigerated at a temperature of 0—5°C. To this solution a quantity of 10 g. of the chlorinated resin prepared as in Procedure 1 was added and 24 ml. of 20% NaOH were slowly added up to a pH value of 11—12, while the mixture was stirred and refrigerated. After 14 hours, the resin was filtered off and washed according to Examples 8. When analyzed, the resulting

resin acylated amine showed 15.9 mg. of mono-linked m-phenylenediamine per gram.

PROCEDURE 10

A quantity of 10 g. of chlorinated resin prepared according to Procedure 3 was poured into a solution of di-o-anisidine 5.25 g. in 75 ml. of dry toluene heated to 80°C. The obtained suspension was heated to 80°C. for 14 hours. After this time the suspension was filtered and the resulting resin washed as in Procedure 8. The resin acylated amine so obtained showed an amount of 63 mg. mono-linked di-o-anisidine per gram.

PROCEDURE 11

An amount of 10 g. of chlorinated resin obtained as in Procedure 3 and a quantity of 5.95 g. of o-tolidine dihydrochloride were reacted according to the conditions reported in Procedure 9. The resulting resin acylated amine contained 11.8 mg of mono-linked o-tolidine per gram.

PROCEDURE 12

A quantity of 10 g. of chlorinated resin obtained as in Procedure 3 and an amount of 5.3 g. benzidine dihydrochloride were reacted according to the conditions in Procedure 9. The obtained resin acylated amine contained 45 mg. of mono-linked o-tolidine per gram.

EXAMPLE 13

A quantity of 3 g. of the resin acylated amine of Procedure 6 was suspended in 10 ml. of 3N HBF_4 and cooled at a temperature of 0—5°C. The resulting suspension was stirred while adding dropwise 10 ml. of 1N $NaNO_2$. Stirring was continued and the temperature maintained for 4 hours. The mixture was then centrifuged and the isolated resin acylated amine diazonium salt,

$$\text{Resin-CO—NH—Ar—}\overset{\oplus}{\text{N}}\text{=N}\ \ \overset{\ominus}{\text{BF}_4}$$

was dried in a vacuum. This method can be extended using the resin acylated amines prepared in Procedures 7—12.

EXAMPLE 14

Resin acylated amine diazonium salts were reacted with α-naphthol in order to determine the coupling activity and to observe the developed colour. The results using resin acylated amine diazonium salts, prepared by the method of Example 13, from each of the resin acylated amines of Procedures 6—12 are shown in Table 1.

TABLE 1

Amine resin of Procedure	Amine resin type	α-Naphthol fixed, mg./g. resin acylated diazonium salt.	Colour developed
6	Resin-di-o-anisidine	27.21	Medium purple-red
7	Resin-di-o-anisidine	36.00	Deep purple-red
8	Resin-di-o-anisidine	4.25	Light purple
9	Resin-m-phenylenediamine	21.4	Orange
10	Resin-di-o-anisidine	29.2	Medium purple-red
11	Resin-o-tolidine	8.1	Red
12	Resin-benzidine	35.6	Brick red

EXAMPLE 15

A mixture of 8 g. of cellulose fibre (cut to a length of about 3 mm.) and 2 g of

$$\text{Resin-CO—NH—Ar—}\overset{\oplus}{\text{N}}\text{=N}\ \ \overset{\ominus}{\text{BF}_4}$$

from Example 13 was homogenized in 5 l. of water at pH 3—4. The homogenized mixture was allowed to sediment and the cellulose resin layer which formed was pressed into a 25 cm. sheet, which possessed a diazo-coupling activity equal to 1/5 that of the original resin acylated amine diazonium salt.

EXAMPLE 16

Sheet material as per Example 15 was cut into strips 0.5 cm. wide and likewise non-reactive strips of the same size of Eaton-Dikeman 627-65 filter paper were prepared.

The strips of the 2 different types of paper assembled with glue to a plastic waterproof support in order to obtain a sheet having 3 reactive zones alternating with 2 non-reactive zones, the top and bottom part of the sheet being constituted by 2 strips of filter paper 3 cm. wide. The resulting sheet was cut into strips of 0.5 cm. each.

EXAMPLE 17

The sheet prepared according to the procedure of Example 15 was cut into 3 mm. × 30 mm. strips. These were glued to a plastic water-proof support having a graduated scale. At the top and bottom part of the support 2 strips of 3 mm. × 30 mm. filter paper were fixed.

EXAMPLE 18

Strips were prepared according to Examples 16 and 17 except that one 3 cm. filter paper end was replaced with glass fibre paper. This glass fibre end was dipped into a solution having the composition (per ml. of aqueous solution) shown in Table 2.

TABLE 2

Ingredient	Weight, mg.
tris(Hydroxymethyl)amino-methane	200
β- or α-Naphthyl phosphate	20
Magnesium sulphate	0.5
	pH = 10.3

The wet strips were dried in a vacuum.

EXAMPLE 19

The procedure of Example 18 was followed except that the composition (per ml. of aqueous solution) shown in Table 3 was used.

TABLE 3

Ingredient	Weight, mg.
Sodium fumarate	100
β- or α-Naphthyl phosphate	20
Magnesium sulphate	0.5
	pH = 5.05

EXAMPLE 20

The sheet prepared according to Example 15 was glued to a plastic support and cut into disc form.

EXAMPLE 21

Tablets were prepared containing the ingredients shown in Table 4.

TABLE 4

Ingredient	Weight, mg.
tris(Hydroxymethyl)amino-methane	18
Starch	2
β- or α-Naphthyl phosphate	0.2
Polyoxyethylene glycol	2.8
Magnesium sulphate	0.05

The tablets were of a weight varying from 22—25 mg.

Into a suitable test tube were introduced a tablet prepared as above and two drops of water. The tablets disintegrated in 10—15 minutes resulting in a turbid solution. A suspect pathologic serum to be analyzed was then added to the solution in the test tube in a quantity of 0.1 ml. and the tube shaken. After 10 minutes incubation at a temperature of 37°C., one drop of 10% orthophosphoric acid solution was added.

A three-zoned test strip prepared according to Example 16 was dipped into the above solution and after 5 minutes the developed colour was observed to be a purple-red. All three zones had been invaded by the colour indicating that the serum had a value of phosphatase activity of the highest level for which the three-zoned test strip was designed, that is, a pathologic value. A normal serum treated as above gave colour only to the first of the three zones. If an unknown serum were to give colour to the first and second zones it would be considered as borderline.

EXAMPLE 22

The continuous strip prepared according to Example 17 was introduced into the test solution used in Example 21. Five minutes later the extension of coloured area was observed and the number corresponding to the highest level of coloured area on the calibrated strip was recorded. Figures from 1—3 indicate a normal value, from 3—5 a borderline value and higher figures indicate pathologic values.

EXAMPLE 23

Into a test tube were introduced a tablet prepared according to Example 21 and 3 drops of milk to be assayed. After 10 minutes a one-zone test strip prepared according to Example 17 was dipped into the resulting solution. Five minutes later the concentration of phosphatase was read based on the colour development of the strip. Colour development in adequately pasteurized milk, for example, should be limited to the figure 1 of the scale. Higher values indicate an inadequate pasteurization or the presence of adulterating raw milk.

EXAMPLE 24

A three-zone strip prepared according to Example 16 was moistened at the end part containing the substrate with 0.05 ml. of a suspect pathologic serum, as used in Example 21 to be assayed. After 10 minutes the strip was washed with water so that the liberated naphthol reached the developer zone. Five minutes later the concentration of phosphatase was read as described in Example 23.

EXAMPLE 25

A one-zone strip prepared according to Example 18 was moistened at the end part containing the substrate with 0.05 ml. of the milk to be assayed. After 10 minutes the strip was washed with water so that the liberated naphthol reached the developer zone. Five minutes later the concentration of phosphatase was read as described in Example 23.

In summary this invention provides an insoluble polymeric diazonium salt colour developer which can be used in the determination of coupling compounds and related enzyme systems.

WHAT WE CLAIM IS:—

1. A colour developer, insoluble in aqueous media, comprising a polymeric aromatic diazonium salt, the aromatic residues of which contain one or more diazotised amino groups each and are bound by an amide linkage to a cation exchange resin.

2. A product according to Claim 1, wherein the cation exchange resin contains carboxyl groups.

3. A product according to Claim 1, wherein the cation exchange resin contains sulphonic acid groups.

4. A product according to Claim 1, wherein the cation exchange resin contains phosphoric acid groups.

5. A product according to Claim 2, wherein the cation exchange resin is a polymer of methacrylic acid.

6. A product according to any of Claims 1 to 5, wherein aromatic residues are derived from di - *o* - anisidine, *m* - phenylenediamine, *o* - tolidine, or benzidine.

7. A product according to Claim 1, substantially as described.

8. A process for the preparation of a polymeric diazonium salt colour developer, insoluble in aqueous media, as claimed in any of Claims 1 to 7, which comprises reacting a cation exchange resin, which contains acid groups, with a halogenating agent to convert some of the acid groups of said resin to acyl halide groups, reacting the resulting acyl halide groups with an aromatic polyamine to provide a resin containing amide linkages and free aromatic amino groups, and diazotising the free aromatic amino groups to produce a polymeric diazonium salt which is insoluble in aqueous media.

9. A process according to Claim 8 substantially as described.

10. A test device for use in the detection of a compound which can be coupled to a diazonium salt which comprises a carrier containing, or coated with, a polymeric diazonium salt colour developer as claimed in any of Claims 1 to 7.

11. A test device for use in the detection of an enzyme system which will catalyse the liberation of a compound, which can be coupled to a diazonium salt, from a substrate for said enzyme system, which comprises a carrier containing, or coated with, a plurality of non-contiguous zones at least one of said zones containing a suitable substrate for said enzyme system and at least one of the remaining zones containing a polymeric diazonium salt colour developer as claimed in any of Claims 1 to 7.

12. A test device as claimed in Claim 10 substantially as described.

13. A method for detecting a coupling compound which comprises adding to a liquid in which said compound, which can be coupled to a diazonium salt, may be present an insoluble polymeric diazonium salt colour developer as claimed in any of Claims 1 to 7, and observing any colour development due to diazo coupling between said polymeric diazonium salt colour developer and any coupling compound which may be present.

14. A method for detecting an enzyme system which will catalyse the liberation of a compound, which can be coupled to a diazonium salt, from a substrate for said enzyme system, which comprises adding to a liquid medium in which said enzyme system may be present a suitable substrate for said enzyme system, adding to the resulting mixture a polymeric diazonium salt colour developer as claimed in any of Claims 1 to 7, and observing any colour development due to diazo coupling between said polymeric diazonium salt colour developer and any coupling compound released from the substrate by any enzyme present.

15. A method according to Claim 14, wherein the enzyme system is lipase, acetyl esterase, N-acetylbetaglucosaminidase, transaminase, leucine aminopeptidase, acid phosphatase or basic phosphatase.

16. A method according to Claim 14 and 15, wherein the substrate is an aromatic phosphate.

17. A method according to Claim 13 or 14 substantially as described.

J. A. KEMP & CO.,
Chartered Patent Agents,
14 South Square,
Gray's Inn, London, W.C.1.

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